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Author(s)	Nomura, Nobuo; Tabata, Satoshi
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A Simple Method for Constructing Cloning Vehicles with Long Polylinker Sequences

Nobuo NOMURA* and Satoshi TABATA**

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A rapid method for constructing new cloning vectors was described. The special features taken for mutagenesis and selection are as follows : (i) mutagenesis was carried out by hybridizing oligonucleotides to single-stranded (SS) template DNA ; (ii) oligonucleotides were designed to harbor short stretches complementary to SS DNA at their both ends ; (iii) SS DNA for template was prepared from the BW313 strain (*dut⁻ ung⁻*) and transformation following hybridization and repair was carried out in JM109 strain (*dut⁺ ung⁺*) in order to enrich an objective construct; (iv) blue-colored colonies (plaques) were selected in the X-gal plates among white-colored colonies (plaques) which are the starting clones and the integrity of the polylinker region was confirmed by sequencing. We describe the construction of new cloning vehicles derived from pUC118 and mp18, and discuss general application of this method to construction of new cloning vectors.

KEY WORDS: Cloning vector / Polylinker / Loop-out mutagenesis

INTRODUCTION

Various cloning vehicles have been developed to date¹⁻⁵⁾. pBR322, one of the representatives, was constructed by attaching two antibiotic resistance genes to the replicon so that recombinants were detected by elimination of one of the drug resistance¹⁾. Polylinkers, DNA sequences retaining the recognition sites for multiple restriction enzymes, were first introduced by Messing and co-workers, and isolation of recombinants was facilitated by change of color of colonies (plaques) from blue to white in the presence of X-gal and IPTG²⁾. Plasmid vectors carrying origins of replication derived from single-stranded bacteriophages have been constructed and this allows copies of one of the two strands of the plasmid DNA to be produced^{3, 5)}. A variety of loop-out mutagenesis protocols has been reported⁶⁻⁹⁾. This type of mutagenesis does not require the naturally occurring restriction sites which are rather limited. Therefore deletions and insertions can be generated at any defined locations. We applied this method, together with Kunkel's method¹⁰⁾, to construct new cloning vehicles which have numerous restriction sites and discuss application to construct various new cloning vehicles.

* 野村信夫: Laboratory of Molecular Biology, Institute of Gerontology, Nippon Medical School, Kawasaki, Kanagawa 211, Japan

** 田畑哲之: Department of Biology, Faculty of Science, Nagoya University, Nagoya 464-01, Japan

EXPERIMENTAL

Bacterial strains, plasmid, phage and enzymes. E.coli BW313 (*dut-1 ung-1 thi-1 relA1 spoT1/F⁺lysA*) was used for preparation of SS DNA as a template for mutagenesis. The strain for transformation was E.coli JM109 [*F⁺ traD36 lacFZΔ M15 proA⁺B⁺ /recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ (lac-proAB)*]. The plasmid pUC118, M13mp18, restriction endonucleases, T4 kinase and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd..

Table 1. List of Oligonucleotides Used

A	122mer	5'	<u>GTAAAACGACGGCCAGT</u> ATGCATGGTACCGCGGGCCCTGCA GAGCTCGAGACTAGTACTCTAGATATCGTACGCGTCGACCC GGGATCCATGGAGGCCTTAAGCTTCGAATTCGTAATCATG	3'
B	25mer	5'	<u>GACTGGTGAGTATTCAACCAAGTCA</u>	3'
C	44mer	5'	<u>CTCGAGACTAGTACTGGAGATCTAGACATCGATATCGTACG</u> <u>CGT</u>	3'
D	73mer	5'	<u>GACATCGATATCGTACGAATTCGAAGCTTAAGGCCTCCATG</u> <u>GATCCCGGGTCGACGCGTAATCATGGTCATAG</u>	3'
E	33mer	5'	<u>GCTATTTTGTGAGAGATCATCGATGAACGGT</u>	3'
F	25mer	5'	<u>GCAAACAAGAGAGTCGATAGCAGCA</u>	3'
G	25mer	5'	<u>ACCAATGAAACCGTCGATAGCAGCA</u>	3'

Sequences and sizes of oligonucleotides synthesized (oligos A - G) are shown. The regions which hybridize with SS template DNA are underlined.

Oligonucleotide synthesis and purification. Oligonucleotides (see Table 1 for their sequences) were synthesized by an Applied Biosystems 391 PCR-MATE machine using the β -cyanoethyl phosphoamidite method. The oligonucleotides were purified through acrylamide gels containing 7M urea and Sep-Pak C18 columns (Waters).

Vector construction. Oligonucleotides were phosphorylated at their 5' ends with T4 DNA kinase. SS DNA templates from the pUC118 vectors were generated by superinfection with helper phage M13K07³⁰. The hybridization mixture (10 μ l) contained the following: Tris·HCl (pH7.5), 20mM; MgCl₂, 10mM; NaCl, 50mM; DTT, 1mM; SS DNA, 0.2pmole; oligonucleotide (122mer), 0.01pmole; oligonucleotide (25mer~74mer), 0.5pmole. After heating at 70°C for 10min, the mixture was gradually cooled down to room temperature. Then repair-ligation solution (10 μ l) was added to the hybridization mixture. The repair-ligation solution contained the following: Tris·HCl (pH7.5), 20mM; MgCl₂, 10mM; DTT, 10mM; dATP, dTTP, dGTP, dCTP, 200mM; ATP, 1mM; DNA polymerase I Klenow fragment, 2 units; T4 DNA ligase, 700 units. Incubation was overnight at 16°C. Repaired and ligated DNA was introduced into CaCl₂-treated BW313.

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DNA sequencing. The new constructions were confirmed by sequence analysis. Sequencing was carried out by the dideoxynucleotide chain terminator method with modification (7-deaza dGTP instead of dGTP).

RESULTS

Construction of pNST plasmids. We applied "loop-out mutagenesis method" together with Kunkel's method to rapidly construct new plasmid vectors. Fig.1 summarizes the whole

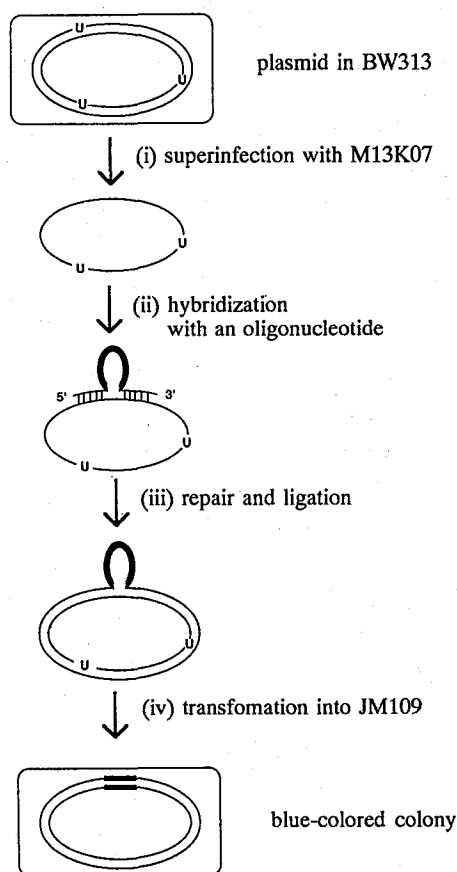


Figure 1. Diagram of producing new vectors. Blue colonies were selected and the integrity of the polylinker sequences was confirmed by sequencing. The region which was inserted by mutagenesis is shown by the bold line. U indicates uracil incorporated.

procedures: (i) SS DNA of the plasmid was produced by superinfection with helper phage M13K07 in BW313 (*dut⁻ ung⁻*); (ii) oligonucleotides which harbor short stretches complementary to SS DNA at their both ends were hybridized; (iii) the repair-ligation reaction was carried out by DNA polymerase I Klenow fragment and T4 DNA ligase; (iv) after transformation into JM109 (*dut⁺ ung⁺*), blue colonies were selected and the integrity of the poly-linker sequences was confirmed by sequencing.

By utilizing this protocol, we produced several vectors which harbor many restriction endonuclease recognition sequences arranged in orientation suitable for the unidirectional deletion method⁽¹¹⁾. Oligo A which is 122base long and contains 24 unique restriction sites and oligo B designed for elimination of the *Sca*I site of pUC118 (Table 1) were hybridized with SS DNA derived from a pUC118 derivative which has a 200bp insert at *Eco*RI-*Bam*HI sites and therefore produces white colonies. Half of the colonies produced after transformation of repaired and ligated DNA into JM109 became blue, indicating the oligonucleotide has been replaced at the polylinker region in high efficiency. Digestion with the *Sca*I restriction endonuclease revealed efficient elimination of the *Sca*I site in the Amp gene (data not shown). Two appropriate clones were selected and sequenced. Although one clone has substitution at the *Ava*III site, the other was shown to have the correct polylinker sequence and named pNST1. In order to insert the *Bgl*II and *Cla*I susceptible site into pNST1, oligo C was hybridized to SS DNA of a pNST1 derivative which lost the *Xba*I site due to cleavage followed by repair with the Pol I Klenow fragment and produces white colonies. After transformation of repaired and ligated DNA, we obtained pNST4. pNST5 which has the reverse orientation of the polylinker sequences, in contrast with pNST4, from the *Eco*RI through the *Mlu*I site was also produced by hybridization of oligo D. The nucleotide sequence of the polylinker site of pNST1, 4, and 5 is shown in Fig.2.

Construction of NT phages. SS DNA of an mp18 derivative which has 400bp fragment at *Pst*I and *Eco*RI site was prepared in the BW313 strain and was hybridized with oligos A, E, F and G. Oligo E was used for elimination of the *Bgl*II site and oligos F and G are for exclusion of the *Cla*I sites. After transformation into JM109, about 20% of the plaques appeared became blue in the presence of X-gal and IPTG. 20 plaques were picked and RF DNA was prepared. 10 clones were not cut either *Bgl*II or *Cla*I, indicating that recognition sites of these enzymes were successfully eliminated. To confirm the DNA sequences of the polylinker site of these clones, 4 clones were sequenced and all four were shown to retain proper sequences. One clone among them was named NT1. NT4 and NT5 were made by use of the same oligos as for construction of pNST4 and pNST5 (Fig.2).

Internal translational initiation of the *lac*Z gene: During the course of the work, we notified possible internal initiation of the *lac*Z gene in certain cases. We further studied such function by modifying the restriction sites. Authentic coding frame of *lac*Z of pNST4 and pNST5 was interrupted by cleavage and repair at various restriction sites. Although modification at the *Xho*I, *Pst*I or *Kpn*I site completely abolishes the function to produce blue colonies, interruption at the *Eco*RI, *Hin*dIII, *Bam*HI, *Mlu*I, *Cla*I or the *Bgl*II site does not (data not shown). This result suggests internal translational initiation of the *lac*Z gene in pNST4 and pNST5 plasmid. We tested whether the internal initiation is also observed in the widely

pNST4/NT4

ST4/NT4

Met AsuII AflIII BamHI HincII SphI ClaI BglIII SpeI

5' CAGGAAACAGCTATGACCATGATTACGAATTCGAAGCTTAAAGCCTCCATGGATCCCGGGTCGACGCGTACGATATCGATGTCTAGATCTCCAGTACTA 3'

3' GTCCTTTGTCGATACTGGTACTAATGTTTAAAGCTTCGAATTCGGAGGTACCTAGGGCCCAGCTGCGCATGCTATAGCTACAGATCTAGAGGTCATGAT 5'

SstI ApaI KpnI

XhoI PstI SstII AvaIII

5' GTCTCGAGCTCTGCGAGGGCCGCGGTACCATGCATACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAAC 3'

3' CAGAGCTCGAGACGTCCCGGGCGCCATGTTACGTATGACCGGCAGCAAAATGTTGCAGCACTGACCCCTTTG 5'

3' TGACCGGCAGCAAAATGT5'

-21M13 (ABI)

pNST5/NT5

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ST5/NT5
                                     AccI
                                     HincII
                                     SalI      BamHI      StuI      HindIII EcoRI      ClaI      BglIII      SpeI
Met          MluI      SmaI      NcoI      AflII      AsuII      SplI      EcoRV      XbaI      ScaI
5'  CAGGAAACAGCTATGACCATGATTACGCGTCGACCCGGGATCCATGGAGGCCTTAAGCTTCGAATTCGTACGATATCGATGTCTAGATCTCCAGTACTA 3'
3'  GTCCTTTGTCGATACTGGTACTAATGCGCAGCTGGGCCCTAGGTACCTCCGGAATTCGAAGCTTAAGCATGCTATAGCTACAGATCTAGAGGTCATGAT 5'

SstI      ApaI      KpnI
XhoI      PstI      SstII      AvaIII
5'  GTCTCGAGCTCTGCAGGGCCCCGGGTACCATGCATACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAAC 3'
3'  CAGAGCTCGAGACGTCCCAGGCGCCATGGTACGTATGACCGGCAGCAAAATGTTGCAGCACTGACCCTTTGTG 5'
3'  TGACCGGCAGCAAAATGT5'
-21M13 (ABI)

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Figure 2. DNA sequences of the polylinker regions of pNST1\NT1, pNST4\NT4 and pNST5\NT5. Recognition sites of restriction endonucleases were shown above the sequences. -21M13 (ABI) primer was indicated. "Met" designates the initiation codon of *lac Z*.

used vector pUC118. Cleavage and repair at any restriction sites within polylinker regions to destroy an open reading frame of *lacZ* do not completely abolish the activity to produce blue colonies (data not shown). Therefore internal initiation of the *lacZ* gene seems to be phenomenon generally observed. This would suggest that the chimera harboring inserts, even if an authentic coding frame is interrupted, do not necessarily produce white colonies. Actually we have often observed such chimera (data not shown). Internal initiation of the *lacZ* gene in the pSL300 and other plasmids was also reported¹²⁾.

DISCUSSION

We described here a simple method for constructing new cloning vehicles. In this method, mutagenesis was carried out by hybridizing oligonucleotides to single-stranded template DNA. Therefore enormous effort for synthesis of multiple oligonucleotides can be avoided. Although construction of vectors in double stranded DNA requires the appropriate restriction enzyme site for insertion of new fragment, there is no limitation in the site for mutagenesis. In the example described here, color change from white to blue was used as a maker. But other change without phenotypic difference can be detected by regular hybridization with a labeled oligonucleotide. Progress in oligonucleotide synthesis makes it possible to synthesize long oligonucleotide. Actually we successfully synthesized 100~176mer, including oligo A (data not shown). In case enough DNA is not made, appropriate quantity can be synthesized by the efficiency of the production of the proper construct high. By applying this method, we produced the pNST1, pNST4 and pNST5 plasmid, and NT1, NT4 and NT5, starting from pUC118 and mp18, respectively. These vectors contain many restriction endonuclease recognition sequences arranged in orientation suitable for the exoIII-Mung bean nuclease terminal deletion method. Other useful vectors should be made simply by use of the method described here.

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